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(54) Title: ENZYMATIC PREPARATION OF POLYSACCHARIDES

(57) Abstract

Provided are *in vitro* processes for the production of polysaccharides such as hyaluronic acid, cellulose, polymannuronan, chitin, etc. These processes are carried out under reaction conditions that permit the recycling of nucleotide-phosphates required for the formation of sugar nucleotide precursors employed in the synthesis of these polysaccharides. Also provided are polysaccharides produced via these processes, and pharmaceutical and cosmetic compositions containing these polysaccharides.

ENZYMATIC PREPARATION OF POLYSACCHARIDES

5   **BACKGROUND OF THE INVENTION**

Field of the Invention

The present invention relates to a process for the
preparation of polysaccharides such as hyaluronic acid
(HA) by enzymatic synthesis *in vitro* using a purified
10   protein fraction containing hyaluronate synthase (HAS).

Description of Related Art

Hyaluronic acid is a naturally occurring linear
polysaccharide composed of repeating disaccharide units
15   of glucuronic acid and N-acetylglucosamine linked by
β-1-3 and β-1-4 glycosidic bonds, as shown below:

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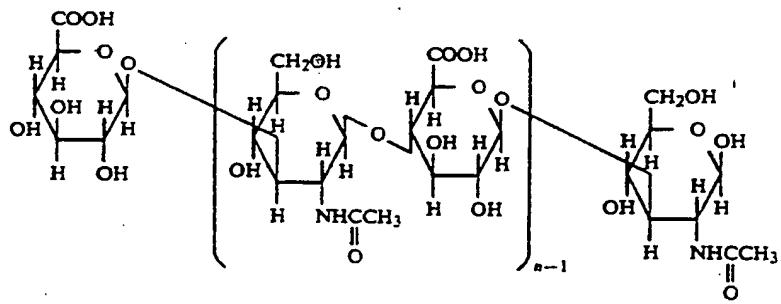
HA is present in all soft tissues of higher organisms, and in particularly high concentrations in the synovial fluid and vitreous humour of the eye (Laurent et al. (1991) <u>Adv. Drug Deliv. Rev.</u> 7:237). In

5   addition to fulfilling structural roles related to its lubricating and water-retaining properties, evidence is mounting that HA also plays an important role in a number of biological processes such as cell motility and cell-cell interactions (see Laurent et al. (1993) <u>FASEB</u>

10   <u>J</u>. 6:2397 and Knudson et al. (1993) <u>FASEB J</u>. 7:1233 for recent reviews).

Since the discovery of HA, much attention has focussed on the possible biomedical applications of highly purified HA fractions. Numerous studies have

15   proven that HA isolated from various sources has an identical chemical structure. Therefore, since it is already present in the human body, exogenously applied HA of sufficient purity is highly biocompatible (i.e., it does not provoke adverse host reactions) and

20   completely biodegradable by natural catabolic pathways. Solutions of purified HA of high molecular weight are extremely viscous and exhibit very interesting rheological behaviour.

As a consequence of the above properties, HA has

25   been widely exploited in the fields of viscosupplementation and viscosurgery (Laurent et al. (1993) <u>FASEB J</u>. 6:2397). Widespread exploitation of HA in other fields where its natural properties might have rendered it suitable has been limited by the fact that

30   unmodified HA exists only in the form of an aqueous gel and has a short residence time upon administration. Therefore, much attention has been focussed on obtaining chemical derivatives of HA which would maintain the biocompatibility of the parent molecule while allowing

35   it to be processed into products for use in areas such as drug delivery and tissue repair (Rastrelli et al.

(1990) <u>Clinical Implant Materials</u> 199; Balazs et al.
(1991) <u>Blood Coagulation Fibrinolysis</u> 2:173).

Sources of Hyaluronic Acid

5   In order to fulfill the raw material requirements
for products for the biomedical applications described
above, the identification of dependable and economically
viable sources of HA has been an industrial priority.

         Initially, attention was focussed on the extraction
of HA from animal tissues. Numerous tissue sources,
10   including umbilical cord, skin and rooster combs, have
been evaluated. Subsequently, rooster comb HA became
the most widely used and traditionally accepted source
both from an industrial and regulatory point of view.
However, there are certain drawbacks to dependence on
15   this source of HA. High molecular weight material is
difficult and costly to isolate due to the fact that the
HA is complexed with proteoglycans. Additionally,
animal-sourced materials for biomedical applications are
coming under increasingly stringent regulatory control
20   due to the fear of contamination with both conventional
and unconventional viral agents. Finally, if HA-based
products become commonly used in sectors such as drug
delivery and tissue repair, predictions indicate that
rooster comb supplies will be insufficient to meet the
25   demand for HA. Therefore, attention has turned in
recent years to the identification of alternative HA
sources.

         Lancefield's group A and C streptococci, which are
human and animal pathogens, respectively, produce HA and
30   have been exploited for the development of industrial-
scale fermentation processes (for example, US patents
4,784,990 and 4,517,295). The equivalence of
streptococcal and rooster comb HA has been demonstrated,
and the former has now been accepted from a regulatory
35   point of view. Supplies of HA from streptococcal
fermentation are theoretically limitless, and no fears

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of seasonal fluctuations or batch-to-batch variations
exist if a tightly controlled process is used. The HA
is produced as a capsule of high molecular weight
material which is easily separated from the micro-
5   organisms, although extensive purification is still
necessary in order to obtain material of sufficient
purity. The possibility of contamination with viral
agents is obviously not a major concern, and the use of
non-pathogenic mutants in the industrial processes
10 ensures the absence of other toxic impurities.

An added advantage of using streptococci, which has
become apparent in recent years, is that recombinant DNA
technology can be applied to these organisms.
Therefore, the tools of molecular biology can be
15 exploited to facilitate the development of a clear
understanding of the molecular mechanisms of HA
synthesis and to allow intervention by genetic
engineering to modulate this process.

HA Biosynthetic Pathway

20   An important step in opening up the possibilities
of genetic engineering is a definition of the HA
biosynthetic pathway. Although no detailed genetic work
has been carried out to identify each step in the
biosynthetic pathway in streptococci, the succession of
25 biochemical events can be put together based on
knowledge of the biosynthesis of the two UDP-sugar
precursors of HA gained from studies carried out in
other organisms (Brede et al. (1991) J. Bacteriol.
173:7042; Mengin-Lecreuix et al. (1993) J. Bacteriol.
30 175:6150) and from some limited streptococcal studies
(Matsubara et al. (1991) Chem. Pharm. Bull. 39:2446).

The proposed biosynthetic pathway for HA is shown
in Figure 1. The precursors, UDP-GlcA and UDP-NAcGlc,
are synthesized as side reactions of the glycolytic
35 pathway starting from glucose-6-phosphate and fructose-
6-phosphate, respectively. Experiments carried out with

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radioactive glucose (O'Regan, unpublished data) have
shown that about 5-7% of the glucose in the
streptococcal culture medium is converted to HA.
Metabolic engineering approaches designed to increase
5   the flux towards HA could be fruitful in terms of
obtaining yields that are more industrially viable.

Hyaluronic Acid Synthase

Much attention has been focussed on hyaluronic acid
synthase as a key enzyme in the biosynthetic pathway of
10 HA and an essential element in developing an
understanding of the mechanism of synthesis.

HA synthase is located in the plasma membrane.
This has been shown by a number of different workers
using various approaches. Markovitz and Dorfman ((1962)
15 J. Biol. Chem. 238:273) first synthesized HA using
streptococcal membranes.   Prehm ((1983) Biochem J.
211:181 and 191) demonstrated the extracellular growth
of the HA chain and that HA was not synthesized in the
Golgi apparatus as are other members of the
20 glycosaminoglycan family. Subsequently, Prehm ((1984)
Biochem J. 220:597) showed that, in the presence of
added UDP precursors, HA synthesis was ten times greater
in disrupted F9 cells as compared to intact cells due to
the increased accessibility of the precursor pool. This
25 suggests that the active domain of the HA synthase may
be located on the internal surface of the plasma
membrane as has been recently hypothesized for the
bacterial enzyme (DeAngelis et al. (1993) J. Biol. Chem.
268:19181).
30 Many attempts have been made to identify the
peptide components of both the prokaryotic and
eukaryotic HA synthases. In streptococci, Prehm et
al.((1986) Biochem. J. 235:887) found proteins of 75,
52, 47, 42 and 34kDa in active HA synthase preparations.
35 Photoaffinity labelling (Van de Rijn et al. (1992) J.
Biol. Chem. 267:24302) revealed three proteins of 42, 33

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and 27kDa which could bind UDP-GlcA. In eukaryotes, a number of different groups have identified active complexes containing several peptide components ranging in size from 52 to 116kDa (Mian (1986) Biochem J.

5   237:333 and 343; Ng et al. (1989) J. Biol. Chem. 264:11776; Klewes et al. (1993) Biochem J. 290:791). It can be concluded from the work described above that a complex of plasma membrane-bound proteins is involved in the synthesis and transport of HA in both prokaryotes

10 and eukaryotes. However, none of the above approaches allowed clear identification of the HA synthase. Recent work in streptcocci, involving a tranposon mutagenesis approach, has resulted in identification of the prokaryotic HA synthase as a 42kDa membrane protein.

15 This significant advance should permit an improvement in the understanding of the molecular mechanisms of HA synthesis in streptococci and a subsequent transposition of this knowledge to eukaryotic organisms.

A number of unusual features of HA synthesis have

20 been revealed in the course of studies by various laboratories. If one protein is responsible for HA synthesis, then the synthase must contain independent binding sites for each substrate. Polymerization is rapid, having been estimated using membrane fractions at

25 aproximately 100 sugar units per minute in eukaryotes (Prehm (1983) Biochem J. 211:181). HA synthesis appears to occur by alternate transfer of the growing chain to UDP-GlcA and UDP-NAcGlc. Unusually, chain growth seems to occur at the reducing end (Prehm (1983) Biochem. J.

30 211:181). Despite numerous attempts, no evidence has been obtained for the involvement of a lipid intermediate in HA synthesis (Sugahara et al (1979) J. Biol. Chem. 254:6252), as is also the case for cellulose synthesis in Acetobacter xylimon (Whitfield (1988) Can.

35 J. Microbiol. 34:415).

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Previous Attempts at *In Vitro* Synthesis of Hyaluronic Acid

Hyaluronic acid was first synthesized from the activated sugar precursors UDP-GlcNAc and UDP-GlcA using
5 cell extracts (Glaser et al. (1955) Proc. Natl. Acad. Sci. USA 41:253). A soluble and particulate form of the enzyme was found in the vitreous humor (Oesterlin et al. (1968a and 1968b) Exp. Eye Res. 7:497 and 511; Oesterlin (1968) Exp. Eye Res. 7:524; Oesterlin (1969) Exp. Eye
10 Res. 8:27; Jacobson (1978a and 1978b) Exp. Eye Res. 27:247 and 259). The synthase was also characterized in fibroblast fractions (Appel et al. (1979) J. Biol. Chem. 254:12199). A promising approach was the analysis of streptococcal protoplast membranes (Markowitz et al.
15 (1962) Meth. Enzymol. 5:155; Stoolmiller et al. (1969) J. Biol. Chem. 244:236; Sugahara et al. (1979) J.Biol. Chem. 254:6252), which led to a proposed mechanism of HA synthesis (Prehm (1983a) Biochem. J. 211:181; (1983b) Biochem. J. 211:191; (1984) Biochem. J. 220:597).
20 Indeed, it was demonstrated that HA synthase resided in plasma membranes (Philipson et al. (1984) J. Biol. Chem. 259:5017; Prehm (1984) Biochem. J. 220:597).

Several attempts were undertaken to identify and isolate HA synthase. Mian ((1986) Biochem J. 237:333
25 and 343) tried to dissolve plasma membranes with the detergent NP-40; Ng et al. ((1989) J. Biol. Chem. 264:11176) dissolved plasma membranes with the mild detergent digitonin. In both cases, active fractions were obtained, which contain several proteins. Prehm
30 et al. (1986) and Triscott et al. (1986) tried to isolate the synthase by solubilization of streptococcal membranes with digitonin and cholate. In 1992, the synthase was identified as a 42 kDa protein using photoaffinity-labelled UDP-glucuronic acid (van de Rijn
35 et al. (1992) J. Biol. Chem. 267:24302). After cloning of HA synthase by DeAngelis et al. (1992) and Dougherty

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et al. ((1993) <u>J. Exp. Med</u>. 175:1291), antibodies were raised against peptides of the HA synthase and subsequently these antibodies were used to purify the enzyme complex from solubilized streptococcal membranes

5   and from recombinant microorganisms (DeAngelis et al. (1994) <u>Biochem</u>. 33:9033). Purification of the streptococcal HA synthase to homogeneity was achieved by Prehm ((1995) Oral presentation, SFB Meeting, Munster, FRG) by solubilization of membranes with digitonin and

10   phase separation with polyethylenglycol.
JP 02-231093 discloses the synthesis of hyaluronic acid by continuous enzyme reaction using 5'-UMP or its salts.

Preparation of Hyaluronic Acid Oligosaccharides

15   Up to the present time, HA oligosaccharides have been prepared by digestion of high-molecular weight HA with hyaluronidases and fractionation using standard chromatographic techniques such as those described by West et al. ((1989) <u>Exp. Cell Res</u>. 183:176-196) and EP

20   88 305255.7. By these known methods, it is only possible to isolate HA oligosaccharides having a molecular weight within the range of 2-4 kDa, i.e., highly polydispersed, and it is not possible to establish the starting and terminating saccharide unit

25   beforehand.

SUMMARY OF THE INVENTION

Recent advances in understanding of the HA biosynthetic apparatus have opened up the possibility of

30   developing a system for *in vitro* enzymatic synthesis of HA. As far as the present inventors are aware, no workers in the field have proposed the possibility of producing HA *in vitro* on an industrial scale. Furthermore, this would be commercially inviable if UDP-

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sugars were used as starting materials as they are prohibitively expensive. An innovative feature of the present invention is the use of biochemical recycling reactions to generate UDP-sugars *in situ*, a feature previously undescribed in the field of polysaccharide biosynthesis.

As discussed above, reliable sources of HA exist and are extensively exploited in industry. However, despite the extensive purification carried out on the polymer, concerns are still being expressed about the possibility of contamination with unknown agents such as non-conventional viruses. Additionally, as a consequence of this extensive purification, a polymer of considerable molecular weight polydispersity is obtained. *In vitro* enzymatic synthesis would permit a production of a polymer of extremely high purity and optimized physico-chemical characteristics. The latter properties could be optimized by synthesizing a polymer of the desired molecular weight with a minimized molecular weight polydispersity. Optimization of the technology could also permit synthesis of monodisperse HA oligosaccharides which might demonstrate improved biological activity when compared to the oligosaccharide fractions obtained by hyaluronidase digestion of HA followed by chromatographic separation. In the longer term, the optimization of *in vitro* technology could permit synthesis of novel polymers by modifying the catalytic site of the synthase to obtain enzymes which combine the sugar moieties in varying ways or incorporate alternative sugar components.

The ability to isolate sufficient quantities of highly purified, active HAS allows the development of an efficient synthetic method for the *in vitro* production of HA. HA produced by such a system would offer considerable advantages over the currently available products obtained from animal or bacterial sources.

For example, by using immobilized HAS, it is

possible to produce HA that is free from contaminating
proteins or infective agents.   By varying the time
and/or conditions of the reaction, it is possible to
produce a polymer of specific molecular weight,thus
5   facilitating the synthesis of HA of various molecular
weights depending upon the intended applications. The
ability to regulate the reaction conditions makes it
possible to control the polydispersity of the molecular
weight, and thus produce a far more homogeneous product
10   than is currently available. Moreover, by employing
immobilized HAS, it is possible to produce HA oligo-
saccharides having defined molecular weights.   By
incubating the immobilized enzyme alternately with the
UDP precursors UDP-glucuronic acid (UDP-GlcA) and
15   UDP-N-acetylglucosamine (UDP-NAcGlc), it is possible to
control the molecular weight of the HA oligosaccharides.

     It is therefore an object of the present invention
to provide a process by which it is possible to obtain
hyaluronic acid free from contaminants such as proteins,
20   viruses, and other impurities normally present in this
polymer when obtained by conventional extraction
methods.

     Another object of the present invention is to
provide a method for purifying and isolating a protein
25   fraction possessing HAS activity.

     Another object of the present invention is to
provide a method of immobilizing HAS.

     Another object of the present invention is to
provide a process for producing HA in vitro with
30   different molecular weights, comprising:

     incubating a protein or protein mixture active in
synthesizing hyaluronic acid in a reaction mixture
containing uridine-5'-triphosphate, glucose-1-phosphate,
and N-acetylglucosamine-1-phosphate for a time and under
35   reaction conditions suitable for the polymerization of
UDP-glucuronic acid and UDP-N-acetylglucosamine to form
hyaluronic acid, and suitable for the formation of UDP-

glucuronic acid from UDP and glucose-1-phosphate and the
formation of UDP-N-acetylglucosamine from UDP and
N-acetylglucosamine-1-phosphate; and

　　　　recovering said hyaluronic acid thus produced.

5　　　　Another object of the present invention is to
provide a process for producing hyaluronic acid *in
vitro*, comprising:

　　　　incubating a protein or protein mixture active in
synthesizing hyaluronic acid in a reaction mixture
10　suitable for the synthesis of hyaluronic acid from UDP-
glucose and UDP-N-acetylglucosamine for a time and under
conditions suitable for the polymerization of UDP-
glucuronic acid and UDP-N-acetyl-glucosamine to form
hyaluronic acid, and suitable for the formation of UDP-
15　glucuronic acid from UDP and glucose-1-phosphate and for
the formation of UDP-N-acetylglucosamine from UDP and N-
acetylglucosamine-1-phosphate,

　　　　wherein within said reaction mixture, uridine-
5'-triphosphate, glucose-1-phosphate, and N-acetyl-
20　glucosamine-1-phosphate are contained within a single
hollowfiber, and

　　　　recovering said hyaluronic acid thus produced.

　　　　Yet another object of the present invention is to
provide a process for producing hyaluronic acid *in
25　vitro*, comprising:

　　　　incubating a protein or protein mixture active in
synthesizing hyaluronic acid in a reaction mixture
suitable for the synthesis of hyaluronic acid from
UDP-glucose and UDP-N-acetylglucosamine,

30　　　　wherein within said reaction mixture, uridine-
5'-triphosphate and glucose-1-phosphate are contained
within a first hollowfiber, and uridine-5'-triphosphate
and N-acetylglucosamine-1-phosphate are contained within
a second hollowfiber,

35　　　　wherein reaction conditions in said first
hollowfiber are suitable for the formation of UDP-
glucuronic acid from uridine-5'-triphosphate and

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glucose-1-phosphate, and wherein reaction conditions in said second hollowfiber are suitable for the formation of UDP-N-acetylglucosamine from uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, and

5   recovering hyaluronic acid thus produced.

Another object of the present invention is to provide a process for producing hyaluronic acid *in vitro*, comprising:

incubating an immobilized or non-immobilized
10   protein or protein mixture active in synthesizing hyaluronic acid in alternate cycles with a mixture of uridine- 5'-triphosphate and glucose-1-phosphate, and separately with a mixture of uridine-5'-triphosphate and N-acetylglucosamine-1-phosphate, in either order,

15   wherein each alternate cycle is for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetyl-glucosamine to form hyaluronic acid,

wherein each alternate cycle is under reaction
20   conditions suitable for the formation of UDP-glucuronic acid from uridine 5'-triphosphate and glucose-1-phosphate, and for the formation of UDP-N-acetylglucos-amine from uridine 5'-triphosphate and N-acetylglucos-amine-1-phosphate, respectively; and

25   recovering hyaluronic acid thus produced.

Another object of the present invention is to provide a process for producing hyaluronic acid *in vitro*, comprising:

incubating an immobilized or non-immobilized
30   protein or protein mixture active in synthesizing hyaluronic acid in alternate cycles with a mixture of uridine-5'-triphosphate and glucose-1-phosphate, and separately with a mixture of uridine-5'-triphosphate and ·N-acetylglucosamine-1-phosphate, in either order,

35   wherein each alternate cycle is for a time and under reaction conditions suitable for the polymerization of UDP-glucuronic acid and UDP-N-acetyl-

glucosamine to form hyaluronic acid,

wherein said uridine-5'-triphosphate and glucose-1-
phosphate are contained within a first hollowfiber under
reaction conditions suitable for the formation of
UDP-glucuronic acid,

said uridine 5'-triphosphate and N-acetylglucos-
amine-1-phosphate are contained within a second
hollowfiber under reaction conditions suitable for the
formation of UDP-N-acetylglucosamine; and

recovering hyaluronic acid thus produced.

A further object of the present invention is to
provide hyaluronic acid produced by any of the foregoing
processes, wherein the molecular weight of said
hyaluronic acid has a polydispersity of between about 1
and about 2.

Another object of the present invention is to
provide a pharmaceutical or cosmetic composition
containing hyaluronic acid produced by any of the
foregoing processes.

Another object of the present invention is the use
of hyaluronic acid produced by any of the foregoing
processes for the preparation of a pharmaceutical or
cosmetic composition.

A further object of the present invention is to
provide a process for producing cellulose *in vitro*,
comprising:

incubating cellulose synthase in a reaction mixture
containing uridine-5'-triphosphate and glucose-1-
phosphate for a time and under reaction conditions
suitable for the synthesis of cellulose, and suitable
for the formation of UDP-glucose from uridine-
5'triphosphate and glucose-1-phosphate; and

recovering cellulose thus produced.

Yet a further object of the present invention is to
provide a process for producing polymannuronic acid *in
vitro*, comprising:

incubating GDP-mannuronic acid polymerase in a

reaction mixture containing guanosine-5'-triphosphate
and mannose-1-phosphate for a time and under reaction
conditions suitable for the synthesis of polymannuronic
acid, and suitable for the formation of GDP-mannuronic

5   acid from guanosine-5'-triphosphate and mannose-1-
phosphate, and

recovering polymannuronic acid thus produced.

A still further object of the present invention is
to provide a process for producing chitin in vitro,

10   comprising:

incubating chitin synthase in a reaction mixture
containing uridine-5'-triphosphate and N-acetylglucos-
amine-1-phosphate for a time and under reaction
conditions suitable for the synthesis of chitin, and

15   suitable for the formation of UDP-N-acetylglucosamine
from uridine-5'-triphosphate and N-acetylglucosamine-1-
phosphate, and

recovering chitin thus produced.

Another object of the present invention is to

20   provide a process for producing a polysaccharide in
vitro, comprising:

incubating the sugar-nucleotide synthase or
polymerizing enzyme for said polysaccharide in a
reaction mixture containing sugar precursors for said

25   polysaccharide and nucleotide triphosphate carriers for
said sugar precursors for a time and under reaction
conditions suitable for the synthesis of said
polysaccharide, and suitable for the formation of sugar-
nucleotide precursors from said nucleotide triphosphate

30   carriers and said sugar precursors, and

recovering said polysaccharide thus produced.

Further scope of the applicability of the present
invention will become apparent from the detailed
description and drawing provided below. However, it

35   should be understood that the detailed description and
specific examples, while indicating preferred
embodiments of the invention, are given by way of

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illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

5   **BRIEF DESCRIPTION OF THE DRAWINGS**

The above and other objects, features, and advantages of the present invention will be better understood from the following detailed descriptions taken in conjunction with the accompanying drawings, all
10   of which are given by way of illustration only, and are not limitative of the present invention, in which:

Figure 1: Shows the proposed biosynthetic pathway for hyaluronic acid.

Figure 2: Shows separation of an extract of
15   digitonin-solubilized membranes of *S. equisimilis* D181 by anion exchange chromatography. Membranes were loaded with HA by incubating with UDP-GlcA and UDP-NAcGlc. After adding PEG and phase separation, the supernatant is loaded on a DEAE column. (Top) SDS-PAGE analysis of
20   the proteins of fractions 10 - 30. (Bottom) elution profile: hyaluronate synthase activity (boxes) determined according to Prehm ((1983) Biochem. J. 211:181 and 191); NaCl gradient (triangles).

Figure 3: Shows HA produced by immobilized HAS.
25   Immobilized HAS (5 mg of protein/g solid support) was incubated in a 10 ml solution containing 100 mM phosphate buffer, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 1 mM UDP-GlcA and 1 mM UDP-NAcGlc for 2 hours at 37°C. HA was liberated from the immobilized HAS by adding NaCl to
30   a final concentration of 1 M. Immobilized HAS was removed by low centrifugal force (500 x g), and the HA was analysed by SDS-PAGE. Electrophorectic conditions and staining are those described by Moller et al. (1993).

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Residual contaminants at the bottom of the gel are probably unincorporated UDP-sugars or very low molecular weight HA.

Figure 4: Shows enzymatic synthesis of hyaluronic
5   acid. The figure demonstrates the incorporation of glucose-1-P in HA using a multienzyme reaction with sugar nucleotide regeneration. The reaction was carried out in 1 ml containing 100 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 4 mM DTT, 50 mM KCl, 10 mM Glc-1-P, 10 mM
10   GlcNAc-1-P, 1 mM UTP, 20 mM PEP, 0.5 mM NAD, 50 LDH, 50 PK, 50 U/ml inorganic pyrophosphatase, 2 U/ml UDP-GlcNAc pyrophosphorylase, 0.5 U UDP-Glc dehydrogenase, 1 U/ml UDP-Glc pyrophosphorylase, and 200 µg HAS for 48 hours at 25°C. The solution was treated with proteinase K and
15   pronase E (see above), and proteins were precipitated by adding TCA to 5% (v/v). The solution was clarified by centrifugation and loaded on a Sepharose CL-4B column, eluted with PBS, and the radioactivity of each fraction was counted. About 90% of the starting amount of Glc-1-
20   P is incorporated in HA.

Figure 5: Shows molecular weight determination by GPC-MALLS of the HA produced in vitro. HA was produced as described in Figure 4, using non-radioactively labelled Glc-1-P. After TCA precipitation of the
25   proteins and centrifugation, the supernatant was dialysed (molecular weight cut-off 5000) extensively against water, lyophilized, resuspended in water, and loaded for GPC-MALLS. 1 = HA of molecular weight of about 600,000; 2 = HA of molecular weight of about
30   540,000; 3 = residual contaminants.

Figure 6: Scheme showing enzymatic synthesis of HA with regeneration of sugar nucleotides.

Figure 7: Scheme showing enzymatic synthesis of HA with regeneration of sugar nucleotides in a hollowfiber
35   enzyme reactor.

Figure 8: Scheme showing enzymatic synthesis of cellulose with regeneration of sugar nucleotides.

Figure 9:   Scheme showing enzymatic synthesis of
polymannuronic   acid   with   regeneration   of   sugar
nucleotides.

Figure 10:   Scheme showing enzymatic synthesis of
5   chitin with regeneration of sugar nucleotides.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description is provided to
aid those skilled in the art in practicing the present
10   invention.   Even so, the following detailed description
should not be construed to unduly limit the present
invention, as modifications and variations in the
embodiments herein discussed may be made by those of
ordinary skill in the art without departing from the
15   spirit or scope of the present inventive discovery.

The contents of each of the references cited herein
are herein incorporated by reference in their entirety.

EXAMPLE 1
Isolation and Purification of Hyaluronic
20   Acid Synthase

In order to achieve in vitro synthesis of HA and to
fully exploit the advantages of the above-described
processes, it is necessary to develop a fast and simple
method for the purification of active HAS.   To purify
25   active HAS, various different approaches can be used,
such as phase separation, affinity chromatography, etc.

HAS can be purified according to the following
protocol:

1.   Streptococcal strains such as Streptococcus
30   equisimilis D181 strain obtained from the Rockefeller
University strain collection, New York, or other cells
capable of producing HAS, such as the species related to
Lancefield's A and C groups; eukaryotic cells; or

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recombinant organisms which express HAS, including
S. zooepidemicus strains, such as the strains 68270 and
68222 (Fidia Research Sud, Siracusa, Italy); *S. pyogenes*
strains, such as the strains S43/192/4; *S. pyogenes* WF50

5   or WF51; a *Pasteurella multocida* Carter type A strain
880; recombinant bacterial strains containing a vector
or vectors encoding HA synthase, such as the plasmid
pPD41, which codes for the streptococcal HA synthase and
UDP-Glc dehydrogenase and an undescribed stretch of

10   streptococcal genomic DNA; bacteria containing such
plasmids, like *Escherichia coli* and *Enterococcus
faecalis* OG1RF; eukaryotic cells, such as fibroblats,
chondrocytes, and virally transformed cells (e.g., SV40
transformed 3T3 fibroblasts and RSV transformed

15   chondrocytes), are grown in the desired volume of
Todd-Hewitt medium, or any other conventional
nutritional medium, at a temperature of between 30°C and
40°C, preferably 37°C, until a final OD_{600nm} of over 0.01
has been reached, preferably about 0.5. One hour before

20   the cells are harvested, a sufficient quantity of
hyaluronidase (H3884 from Sigma Chemical Co., St. Louis,
MO, USA) is added to the culture to degrade the HA
produced and thus facilitate cell harvesting.

       2.   The cells are pelleted by centrifugation at

25   6,000 x g for 15 minutes at 4°C, washed once in a
suitable buffer, such as a standard cold phosphate
buffered saline (PBS), and resuspended in a final volume
of between 1 and 100 ml, preferably 20 ml, of cold PBS
containing approximately 1mM dithiothreitol (DTT).

30   3.   The suspension is treated by procedures that
cause the destruction of the cells, such as sonication
at 0°C, and under conditions that ensure that cell
destruction is total while minimizing inactivation of
the enzyme. Various different sonication conditions can

35   be employed, but the most suitable have proved to be 15
minutes at 120 watts.   Unless otherwise stated,

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operations should be carried out at 4°C.

Alternatively, cells can be disrupted by French press treatment (e.g., 1000 bar); by treatment with glass beads; or by digestion with cell wall degrading enzymes. One gram of cells in 10 ml standard phosphate buffered saline (PBS) solution or in 50 mM $NaHPO_4/KH_2PO_4$, PH 6.9, 10 mM $MgCl_2$, 5 mM DTT, 30% raffinose, can be digested with lysozyme (1 mg/ml, 37°C, 60 min), mutanolysin (50 μg/ml, 37°C, 60 min), muraminidase (0.1 mg/ml, 37° C, 60 min) or phage lysin (50,000 Units, 37°C, 60 min). After digestion of the cell wall, the protoplasted cells can be lysed by resuspending in hypotonic buffers, such as Tris-malonate or 50 mM $NaHPO_4/KH_2PO_4$, PH 6.9, 10 mM $MgCl_2$, 5 mM DTT. Additionally, to ensure lysis after digestion, sonication, treatment with glass beads, or French press passage can be employed.

4.   Cell debris is removed by centrifugation at 10,000 x g for 15 minutes at 4°C, and the supernatant is subjected to ultracentrifugation under conditions that sediment bacterial membranes, preferably 100,000 x g for 30 minutes.

5.   The sediment containing the cell membranes is resuspended by mild sonication, for instance 30 seconds at 20 watts, in a small volume of Tris-malonate buffer, 50 mM, pH 7, or any other suitable buffer containing approximately 1 mM DTT, such as PBS. 50 mM $NaHPO_4/KH_2PO_4$, pH 6.9, 150 mM NaCl, 10 mM $MgCl_2$, 5mM DTT, 10% glycerol, can also be used.

The protein concentration is then determined, and the suspension is diluted in the same buffer at a final protein concentration of between about 1 and 10 mg/ml, preferably about 3 mg/ml.

6.. To the sample resulting from step 5, a mild

detergent such as digitonin or dodecyl maltoside is added to give a final concentration of between 0.5 and 2%, preferably 1%. The suspension is stirred for about one hour at 0°C and then ultracentrifuged at 100,000 x

5   g for 30 minutes, or under similar conditions, to separate membrane fragments.

       7.   The synthase can be separated from the detergent in a number of ways.

       For example, solubilized membranes can be directly

10   loaded on an ion exchange chromatography column.

       Alternatively, HAS can be affinity purified using poly- or monoclonal antibodies against the HA synthase. These antibodies can be immobilized on a solid support.

       A preferred method is that described by Parish et

15   al.   ((1986) _Anal. Biochem._ 156:594-602) whereby separation is achieved by adding polyethylene glycol (PEG) 6000.   To ensure that the synthase actually separates within the aqueous phase, the protein can be "loaded" with newly-synthesized HA to increase its

20   affinity for the aqueous phase.   To this end, the following compounds are added to 5 ml of the supernatant resulting from step 6: between 0.2 and 5 ml of a 50% solution of PEG 6000, preferably 1 ml; 0.05 ml of a solution of 1 M MgCl$_2$, between 0.5 and 5 mg of

25   UDP-N-acetylglucosamine and UDP-glucuronic acid, preferably 2 mg of each.   The mixture is then incubated under conditions favouring the synthesis of HA, for example at 37°C for 30 minutes, and is then rapidly cooled to 0°C in an iced salt bath.   A further aliquot

30   of 50% PEG 6000 (1 ml) is then added and the solution is vortexed.   At this point, the suspension will look turbid, signifying that phase separation has occurred. The suspension is again ultracentrifuged, for example at 100,000 x g for 30 minutes at 4°C.

35   8.   The active HAS can be separated from the

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mixture in various ways, including inverse phase
chromatography, such as HPLC. The supernatant should
preferably be passed through an ion exchange HPLC
column, such as a Waters DEAE-protein Pak SWP (7.5 cm x

5   7.5 mm) column, which has previously been equilibrated
with a suitable buffer, such as 50 mM Tris-malonate, pH
7.0, containing a suitable quantity of detergent, such
as digitonin, at a final concentration of between 0.01%
and 1%, preferably 0.5%. The sample is pumped onto the

10 column at a suitable flow rate, preferably 1 ml/min.
Once the supernatant has been loaded, the column is
washed with starting buffer, and proteins can be eluted
by gradually increasing the salt concentration of the
buffer, for example from 0 M to 0.5 M NaCl in 30

15 minutes, with a constant flow rate of 1 ml/min.
Fractions can be gathered at a desired volume, but a
volume of 1 ml is advisable.

9.   To identify the fractions containing active
HAS, a series of analyses are performed on each single

20 fraction. These include determining absorbance at 280
nm, conductivity, and HAS activity by means of
radiolabelled precursors, as described by Prehm ((1983)
Biochem. J. 211:181-189). In order to associate the
activity of the HAS with a given protein or protein

25 fraction, the protein pattern of each fraction can be
analysed by polyacrylamide gel electrophoresis,
following precipitation of the proteins from a fixed
volume of each fraction, for example 200 µl, by a
standard technique, such as that described by Wessel and

30 Flügge ((1984) Anal. Biochem. 138:141-143).
The result of a purification experiment is shown in
Fig. 2. HAS peak activity resides in the fractions
containing a substantially pure protein of approximately
42 kDa, corresponding to the HAS described above.

35   These results are consistent with those of
DeAngelis and Wiegel (1992) and Dougherty and van de

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Rijn ((1992) _J. Exp. Med_. 175:1291), who demonstrated
that HAS has a molecular weight of 42 kDa, and further
show that the 42 kDa protein constitutes the active
synthase, and that this protein is sufficient to permit
5   the _in vitro_ synthesis of HA having better
characteristics than the HA which is currently being
isolated from animal or bacterial sources.

EXAMPLE 2

Immobilization of Isolated and Purified
10   **Hyaluronic Acid Synthase**

HAS can be immobilized to solid supports (matrices)
such as those described by Scouten ((1987) _Meth. Enzym_.
135:30-65) and used for the _in vitro_ synthesis of HA.
The following is a typical protocol:

15   1.   In a reaction vessel of the desired size,
isolated and purified HAS is diluted in a coupling
buffer, such as $NaHCO_3$ (0.1 M, pH 8.3) containing NaCl
(0.5 M), or any other suitable buffer such as borate
buffer (0.1M, pH 8.3) containing NaCl (0.5M). It is
20   possible to couple 1 to 10 mg, preferably 5 mg, of
isolated and purified HAS to 1 g of solid support.

2.   The solution resulting from step 1 is mixed
with a solid support, such as CNBr-activated Sepharose
4B (Pharmacia), which has previously been washed with 1
25   mM HCl (200 ml per 1 g of support), or any other
suitable kind of support, and incubated under conditions
that will allow the HAS to be coupled to the support,
for example 16 hours at 4°C using an end-over-end or
similar mixer.
30

3.   The support resulting from step 2 is
transferred into a solution containing a blocking agent
such as 1 M ethanolamine, 0.2 M glycine, or any other

suitable blocking agent, and incubated under conditions that will allow the remaining active groups to be blocked, for instance 2 hours at room temperature.

5   4. The support resulting from step 3 is washed at room temperature with the coupling buffer, or with any other suitable buffer, for example PBS or 50 mM $NaHPO_4/KH_2PO_4$, PH 6.9, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 10% glycerol, to free it from any excess protein and from the blocking agent.

10   5. The support resulting from step 4 is stored at 4°C in a storage solution such as PBS, or any other suitable storage solution.

EXAMPLE 3
In Vitro Production of Hyaluronic Acid
15   ### of Varying Molecular Weight Using
HAS or Immobilized HAS

It is possible to synthesize hyaluronic acid of varying molecular weight for various applications using purified HAS or purified and immobilized HAS. Of
20 particular interest are molecular weight fractions in the range of from about 400 to 50,000 Daltons, and those greater than about 2 x 10^6 Daltons.

As used in the present Example and those that follow, "HAS" refers to pure HAS, a substantially pure
25 protein fraction containing HAS, or a membrane fraction exhibiting HA synthetic activity.

The following is a typical protocol:

1. In a reaction vessel of the desired size, purified HAS or purified and immobilized HAS is diluted
30 in 100 mM HEPES buffer, pH 7.5, or any other suitable buffer, to a final protein concentration of between 0.01 and 1.0 mg/ml, preferably 0.1 mg/ml. Dithriothreitol is

added to the solution to a final concentration of about
1 mM, MgCl$_2$ to a final concentration of about 10 mM,
UDP-GlcA and UDP-NAcGlc, both to concentrations of
between 0.01 and 5 mM, preferably 1 mM.

5   2. The mixture is then incubated at a temperature
of between 30°C and 40°C, preferably 37°C, for long
enough to allow the synthesis of HA, for example, two
hours. The mixture is resuspended in a highly saline
solution, for example 1 M NaCl, so as to allow the
10   release of HA, and the reaction is stopped by removing
immobilized HAS by using low centrifugal forces, for
example 500 x g. When the HAS is not immobilized, the
reaction is stopped by adding proteinase K and pronase
E (from 5 to 100 µg/ml, but preferably 50 µg/ml), and
15   incubating for 30 min at 37° C. The reaction mixture is
then subjected to gel filtration on a Sephadex G-25
column, or any other suitable gel permeation column,
such as Fractogel HW50, Biogel P-4, Biogel P-6, or
Ultrogel AcA202. The HA is eluted with PBS or any other
20   suitable buffer.

          3.   To determine the molecular weight of the HA
produced during steps 1 and 2, radiolabelled precursors
such as those described by Prehm ((1983) Biochem. J.
211:181-189) can be used. Alternatively, the HA product
25   can be analysed by SDS-PAGE, as described by Moller et
al. ((1993) Anal. Biochem. 209:169-175), by GPC-MALLS
(gel permeation chromatography - mid angle laser light
scattering), or by any oher method capable of
determining the molecular weight of HA.
30   The molecular weight of the HA synthesized *in vitro*
by the use of HAS can be varied by modifying the
incubation times and/or reaction conditions. The
reaction conditions that can be varied to influence the
molecular weight of the final HA product include, but
35   are not limited to, pH, temperature, duration, etc. The

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pH can be varied between 4.2 and 8.9; a decrease in the
pH produces a decrease in the molecular weight of the
HA.   The temperature can be varied between 10°C and
40°C; the lower the temperature, the lower the molecular
weight of the HA produced.   The time range for
incubation is between 5 and 120 minutes; shorter
incubation times result in HA of lower molecular weight.
By way of example, HA having a molecular weight of about
500,000 Daltons requires an incubation time of
approximately 30 min at 25°C.

The production of high molecular weight HA by
immobilized HAS is shown in Figure 3.

EXAMPLE 4
Production of HA Oligosaccharides and Polysaccharides
of Controlled Molecular Weights

It is possible to synthesize HA oligosaccharides
and polysaccharides having predefined molecular weights,
preferably from about 400 to about 50,000 Daltons, and
with given starting and terminating saccharide units,
using immobilized HAS and the UDP-precursors UDP-GlcA
and UDP-NAcGlc (hereafter designated "A" or "B",
respectively).   Depending upon the desired initial or
terminal saccharide unit, the first (or last) reaction
step is started (terminated) with one of the two
UDP-precursors A or B.   The molecular weight of the
hyaluronic acid oligosaccharide or polysaccharide can be
controlled by varying the number of times the
immobilized HAS is incubated with UDP-GlcA or
UDP-NAcGlc.

The following is a typical protocol:

1.   In a reaction vessel of the desired size,
immobilized HAS is diluted in 100 mM HEPES buffer, pH
7.5, or in any other suitable buffer, to a final
concentration of between 0.1 and 10.0 mg/ml, preferably

1.0 mg/ml. To this suspension is added dithiothreitol
to a final concentration of about 1 mM and MgCl$_2$ to a
final concentration of about 10 mM. Then a
UDP-precursor (A or B) is added to the suspension to a
5   final concentration of between 0.01 and 5 mM, preferably
1 mM.

2.   The mixture is incubated under suitable
conditions, for example at 25°C for 5 minutes.

3.   The mixture resulting from step 2 is washed
10   with 100 mM HEPES buffer, pH 7.5, or any other suitable
buffer, on a porous glass filter (e.g., with a porosity
of G3), and the matrix is recovered.

4.   The matrix is resuspended in 100 mM HEPES
buffer, pH 7.5, or in any other suitable type of buffer,
15   to a final protein concentration of between 0.1 and 10.0
mg/ml, preferably 1.0 mg/ml. To this suspension are
added dithiothreitol to a final concentration of about
1 mM and MgCl$_2$ to a final concentration of about 10 mM.
Lastly, the alternate UDP-precursor (A or B) is added to
20   a final concentration of between 0.01 and 5 mM,
preferably 1 mM.

5.   The subsequent cycles of incubation, washing,
matrix recovery, and resuspension are the same as those
described in steps 2, 3, and 4, but the UDP-precursors
25   A and B are alternated from cycle to cycle.

6.   Once the desired number of cycles has been
completed, the mixture is washed with HEPES, or any
other suitable buffer, on a porous glass filter (e.g.,
porosity G3), and the matrix is recovered.

30   7.   The matrix is resuspended and incubated in a
highly saline solution, so as to facilitate the release

of the HA oligosaccharide chain from the immobilized
HAS, for example in NaCl (1 M) for 30 minutes at 25°C.
The matrix is then washed again on a porous glass
filter.

5   8. The filtered product is then subjected to gel
filtration on a Sephadex G-25 column or any other
suitable gel filtration medium such as Fractogel
HW50, Biogel P-4, Biogel P-6, or Ultrogel AcA202. The
HA is eluted with PBS or any other suitable buffer.

10   9. In order to determine the molecular weight of
the HA oligosaccharides produced according to steps 1 to
8, radiolabelled precursors can be used, as described by
Prehm ((1983) _Biochem. J_. 211:181-189). Alternatively,
the HA thus produced can be analysed by SDS-PAGE, as
15 described by Moller et al.   ((1993) _Anal. Biochem_.
209:169-175), GPC-MALLS, or by any other method capable
determining the molecular weight of HA.

EXAMPLE 5
Recycling of UDP and Enzymatic Regeneration
20   ### of UDP-GlcA and UDP-NAcGlc During the
In Vitro Production of Hyaluronic Acid

UDP can be recycled, and the UDP-precursors
UDP-glucuronic acid (UDP-GlcA) and UDP-N-Acetyl-
glucosamine (UDP-NAcGlc) employed in _in vitro_ HA
25 synthesis can be enzymatically regenerated, starting
from   glucose-1-phosphate   (Glc-1-P)   and
N-Acetylglucosamine-1-phosphate   (NAcGlc-1-P),
phosphoenolpyruvate (PEP), and catalytic quantities of
NAD^+ and UDP. Similar methods have been described by
30 Ichikawa et al.   ((1992) _Anal. Biochem_. 202:215-238),
Hindsgaul et al.   ((1991) _Enzymes in Carbohydrate
Synthesis_, ACS Symposium Series 466:38-50), and Gygax et
al. ((1991) _Tetrahedron_ 47:5119-5122).

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The following is a typical protocol:

1.   In a reaction vessel of the desired volume, purified HAS or purified and immobilized HAS is diluted in a suitable buffer, for example 100 mM HEPES buffer, pH 7.5.

2.   To the solution resulting from step 1, the following reagents are added to concentrations of approximately: DTT (between 0.1 and 10 mM, preferably 4 mM), $MgCl_2$ (between 0.1M and 5mM, preferably 10 mM), KCl (between 0.1 M and 100 mM, preferably 50 mM), UTP (between 0.01 and 1 mM, preferably 0,1 mM), NAD^+ and NADH (each between 0.01 and 1 mM, preferably 0.1 mM), Glc-1-P and NAcGlc-1-P (each between 2 mM and 100 mM, preferably 10 mM), PEP (between 2 and 50 mM) pyruvate kinase (PK) and lactate dehydrogenase (LDH) (each between 10 and 1000 U/ml, preferably 50 U/ml), inorganic pyrophosphorylase (PPase) (between 1.2 and 120 U/ml, preferably 50 U/ml), UDP-Glc pyrophosphorylase (between 0.1 and 10 U, preferably 1 U), UDP-GlcNAc pyrophosphorylase (between 0.1 and 20 U, preferably 2 U), and UDP-Glc dehydrogenase (between 0.1 and 10 U/ml, preferably 1 U/ml). The enzymes can also be immobilized on solid supports as described in Example 2, or separated from the HAS by compartmenting them, for example by entrapping them in a hollowfiber system.

3.   The solution resulting from step 2 is incubated under suitable reaction conditions, preferably for 48 hours at 25°C, in order to reduce the risk of oxidation of the reaction components, preferably under anaerobic conditions, for example under argon or nitrogen.

The reaction is stopped by removing the enzymes by using low centrifugal forces, for example 500 x g, when they are immobilized, or by removing the compartmenting system, for example by removing the hollowfiber system,

when the enzymes are entrapped within a hollowfiber.

When the enzymes are not immobilized, the reaction
is stopped by adding proteinase K and pronase E (from 5
to 100 μg/ml, but preferably 50 μg/ml), and incubating
5   for 30 min at 37°C.

4.   The solution resulting from step 3 is
chromatographed on a Sephadex G-25 gel permeation
column, or any other suitable gel permeation medium such
as Fractogel HW50, Biogel P-4, Biogel P-6, or Ultrogel
10   AcA202. The HA is eluted with PBS or any other suitable
buffer.

5.   To determine the molecular weight of the HA
produced according to steps 1, 2 and 3, labelled
precursors can be used, such as those described by Prehm
15   ((1983) <u>Biochem. J</u>. 211:181-189). Alternatively, the HA
thus produced can be analysed by SDS-PAGE, as described
by Moller et al. ((1993) <u>Anal. Biochem</u>. 209:169-175),
GPC-MALLS, or by any other method capable determining
the molecular weight of HA.
20   The results of the incorporation of radioactively
labeled Glc-1-P in HA are shown in Figure 4.
         The results of the molecular weight determination
by GPC-MALLS (gel permeation chromatography - mid angle
laser light scattering) of the HA produced <i>in vitro</i> are
25   shown in Figure 5.
         The HA produced in this way has all the necessary
characteristics   to   be   used   advantageously   in
pharmaceutical   applications   and   other   related
applications,   being   extremely   pure   compared   to
30   hyaluronic acid purified from conventional sources, and
being   free   from   any   significant   quantities   of
contaminating   proteins,   pyrogenic   or   inflammatory
substances, or viruses.

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EXAMPLE 6

In Vitro Production of HA Oligosaccharides and Polysaccharides of Controlled Molecular Weights With Recycling of UDP and Enzymatic Regeneration of UDP-GlcA and UDP-NAcGlc

HA oligosaccharides and polysaccharides of predefined molecular weights in the range of from about 400 Daltons to about 50,000 Daltons, with given starting and terminating saccharide units, can be produced in a manner similar to that described in Example 4, taking advantage of the cost-effectiveness of UDP recycling and enzymatic regeneration of UDP-GlcA and UDP-NAcGlc from the relatively inexpensive precursors Glc-1-P and NAcGlc-1-P, as described in Example 5.

Briefly, immobilized or non-immobilized pure HAS, substantially pure HAS, or a membrane fraction exhibiting HA synthetic activity, can be alternately contacted with UDP-GlcA and UDP-NAcGlc regenerating systems under conditions suitable for HA synthesis. In this way, HA oligosaccharides and polysaccharides of defined molecular weights can be cheaply produced.

In another embodiment exemplifying this approach, HAS can be alternately introduced into UDP-GlcA and UDP-NAcGlc regenerating reaction media as described in Example 5, for example by successively recovering immobilized HAS, or compartmentalizing HAS in, for example, a hollowfiber containing HAS in HA synthesis reaction medium.

By any of the foregoing methods, the molecular weight of the HA oligosaccharide or polysaccharide can be controlled by varying the number of times the HAS is incubated with the UDP-GlcA and UDP-NAcGlc regenerating systems.

The following is a typical protocol:

1.   In a reaction vessel of the desired volume,

purified HAS, or purified and immobilized HAS, is
diluted in 100 mM HEPES buffer, pH 7.5, or in any other
suitable buffer, to a final concentration of between 0.1
and 10.0 mg/ml, preferably 1.0 mg/ml. Dithiothreitol is
5   added to the solution to a final concentration of about
1 mM and $MgCl_2$ to a final concentration of about 10 mM.

2.   Two hollowfiber systems are employed, one
containing the precursors, enzymes, cofactors, etc.,
required for the regeneration of UDP-GlcA; the other
10   containing the precursors, enzymes, cofactors, etc.,
required for the regeneration of UDP-NAcGlc.
The buffer system, precursor concentrations, enzyme
concentrations, cofactor concentrations, etc., are the
same as described in step 2 of Example 5 in the
15   respective hollowfibers.

3.   The first hollowfiber containing the
regeneration system for either UDP-Glc or UDP-NAcGlc, as
desired, is introduced into the reaction vessel, and the
mixture is incubated under suitable conditions, for
20   example at 25°C for 5 minutes.

4.   The first hollowfiber is removed, and the
second hollowfiber containing the alternate
UDP-precursor regeneration system is introduced into the
reaction vessel and incubated as for the first
25   hollowfiber.

5.   Subsequent cycles of hollowfiber introduction
and incubation are the same as those described in steps
3 and 4, alternating from cycle to cycle.

6.   Once the desired number of cycles has been
30   completed, the matrix is recovered from the reaction
vessel by washing on a porous glass filter (e.g.,
porosity G3), and the matrix is recovered.

7. The matrix is resuspended and incubated in a highly saline solution so as to facilitate the release of the HA oligosaccharide chain from the immobilized HAS, for example in NaCl (1 M) for 30 minutes at 25°C. The matrix is then washed again on a porous glass filter.

8. The filtered product is then subjected to gel filtration. Depending upon the molecular weight of the HA produced, different gel filtration media can be used. For HA oligosaccharides with lower molecular weights, a suitable gel filtration medium is Sephadex G-10 or any other similar gel filtration medium such as Biogel P-2 or Sephadex G-15. For larger oligo- or polysaccharides up to 50,000 Daltons, a suitable gel filtration medium is Fractogel HW40 or any other similar gel filtration medium such as Fractogel HW50. The HA is eluted with PBS or any other suitable buffer.

9. When the HAS is not immobilized, the hollow fiber is removed, and the reaction is stopped by adding proteinase K and pronase E (from 5 to 100 µg/ml, but preferably 50 µg/ml) and incubating for 30 min at 37° C. The reaction mixture is then subjected to gel filtration. Depending upon the molecular weight of the HA produced, different gel filtration media can be used. For HA oligosaccharides with lower molecular weights, a suitable gel filtration medium is Sephadex G-10 or any other similar gel filtration medium such as Biogel P-2 or Sephadex G-15. For larger oligo- or polysaccharides up to 50,000 Daltons, a suitable gel filtration medium is Fractogel HW40 or any other similar gel filtration medium such as Fractogel HW50. The HA is eluted with PBS or any other suitable buffer.

10. In order to determine the molecular weight of the HA oligosaccharides and polysaccharides produced

according to steps 1 to 9, radiolabelled precursors can be used, as described by Prehm ((1983) <u>Biochem. J.</u> 211:181-189). Alternatively, the HA thus produced can be analysed by SDS-PAGE, as described by Moller et al. ((1993) <u>Anal. Biochem.</u> 209:169-175), GPC-MALLS, or by any other method capable determining the molecular weight of HA.

<u>EXAMPLE 7</u>

<u>Compartmentalized System With Recycling of UDP and</u>
<u>Enzymatic Regeneration of UDP-GlcA and UDP-NAcGlc</u>
<u>For the *In Vitro* Synthesis of Hyaluronic Acid</u>

HA can also be synthesized using a system wherein UDP is recycled and UDP-GlcA and UDP-NAcGlc are enzymatically regenerated during the synthesis of HA *in vitro* by employing a "two pot system" wherein hollowfibers are employed to compartmentalize the recycling and regeneration reactions.

A typical reaction protocol is as follows.

1.   Two hollowfiber systems are submerged in a reaction solution containing HAS, DTT, and $MgCl_2$, as described in Example 3. One hollowfiber contains the precursors, enzymes, cofactors, etc. required for the regeneration of UDP-GlcA; the other hollowfiber contains the precursors, enzymes, cofactors, etc. required for the regeneration of UDP-NAcGlc. This is schematically shown in Figure 6.

Alternatively, the enzymes required for the regeneration of the UDP-precursors can be contained in a single hollowfiber system which is submerged in an HAS-containing reaction solution.

In either case, the buffer system, precursor concentrations, enzyme concentrations, cofactor concentrations, etc., are the same as described in step 2 of Example 5.

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2.   The reaction solution is incubated under suitable reaction conditions, preferably for 48 hours at 25°C, in order to reduce the risk of oxidation of the reaction components, preferably under anaerobic conditions, for example under argon or nitrogen.

3.   The reaction is stopped by removing the enzymes entrapped within the hollowfiber.

4. The reaction solution containing HAS is treated with proteinase K and pronase E (from 5 to 100 μg/ml, preferably 50 μg/ml), and incubated for 30 min at 37°C. If the HAS is immobilized, the enzyme is removed by using low centrifugal forces, for example 500 x g.

5.   The solution resulting from step 4 is chromatographed on a Sephadex G-25 gel permeation column, or any other suitable gel permeation medium such as Fractogel HW50, Biogel P-4, Biogel P-6, or Ultrogel AcA202. The HA is eluted with PBS or any other suitable buffer.

6.   To determine the molecular weight of the HA produced according to steps 1-5, labelled precursors can be used, such as those described by Prehm ((1983) Biochem. J. 211:181-189).. Alternatively, the HA thus produced can be analysed by SDS-PAGE, as described by Moller et al.   ((1993) Anal. Biochem. 209:169-175), GPC-MALLS, or by any other method capable determining the molecular weight of HA.

EXAMPLE 8
In Vitro Synthesis of Cellulose

Cellulose can be synthesized in vitro using the sugar precursor UDP-glucose (UDP-Glc) and cellulose synthase, which can be isolated from Acetobacter xylinum

or protoplasted plant cells (Wong et al. (1990) <u>Proc.</u>
<u>Natl Acad. Sci. USA</u> 87:8130-8134).

As shown in Figure 8, the reaction system includes
cellulose synthase, and the regeneration system for UDP-
5   Glc includes glucose-1-P, phosphoenolpyruvate, $MgCl_2$,
DTT, pyruvate kinase, UDP-Glc pyrophosphorylase, and
inorganic pyrophosphatase. Reaction conditions are
similar to those described in Example 5.

EXAMPLE 9

10   ### *In Vitro* Synthesis of Polymannuronic Acid

Polymannuronic acid (PM) is an immunostimulating
agent, and much research has been done to manipulate the
fermentation conditions of alginate-producing bacterial
strains such as *Pseudomonas aeruginosa*, *P. fluoreszens*,
15   and *Azotobacter vinelandi*. PM can be synthesized *in*
vitro using GDP-mannuronic acid polymerase (Gacesa et
al. (1990) <u>Psedomonas   Infection   and   Alginates,</u>
<u>Biochemistry, Genetics and Pathology</u>, Chapman and Hall,
London).
20   As shown in Figure 9, the reaction system includes
GDP-mannuronic acid polymerase, and the regeneration
system for GDP-mannuronic acid contains mannose-1-P,
phosphoenolpyruvate, $MgCl_2$, DTT, pyruvate kinase, GDP-
mannose pyrophosphorylase, GDP-mannose dehydrogenase,
25   and inorganic pyrophosphatase. Reaction condition are
similar to those described in Example 5.

EXAMPLE 10

In Vitro Synthesis of Chitin

As shown in Figure 10, chitin can be synthesized *in*
30   *vitro* using the sugar precursor UDP-N-Acetylglucos-amine
(UDP-NAcGlcA) and chitin synthase, which can be isolated
from *Saccharomyces cerevisiae*. Alternatively, a cloned
enzyme from *S. cerevisiae* can be used (Silverman (1989)

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<u>Yeast</u> 5:459-467; Bulawa (1992) <u>Mol. Cell Biol</u>. 12:1764-1776; Cabib et al. (1983) <u>Proc. Natl. Acad. Sci. USA</u> 80:3318-3321).

The regeneration system for UDP-NAcGlcA includes N-acetylglucosamine-1-P, phosphoenolpyruvate, $MgCl_2$, DTT, pyruvate kinase, UDP-NAcGlc pyrophosphorylase, and inorganic pyrophosphatase. Reaction conditions are similar to those described in Example 5.

EXAMPLE 11
PHARMACEUTICAL COMPOSITIONS

Pharmaceutical compositions containing an effective amount of hyaluronic acid prepared according to the methods of the present invention, alone or in association with one or more active principles, pharmacologically acceptable carriers, diluents, or excipients, can be used advantageously in the medical/pharmaceutical field, for example in ophthalmology, in the area of tissue repair, and in rheumatology.

These pharmaceutical compositions can be administered by the topical, intra-articular, ophthalmic, systemic, and peritoneal routes. Such pharmaceutical compositions have been described in European Patent 0 138 572 B1, and in PCT publication WO 92/18543.

These compositions typically contain hyaluronic acid in an amount of from about 0.1 mg/ml to about 100 mg/ml.

For topical administration, these compositions can be in the form of creams, gels, ointments, bandages, and gauzes.

For ophthalmic use, they can be in the form of eye drops, viscoelastic surgical aids, lenses, etc.

For systemic administration, pharmaceutical preparations for intravenous or intramuscular

adminstration are suitable, such as ampoules or vials.

Pharmaceutical compositions containing HA having a molecular weight of from about 400 Daltons to about 50,000 Daltons are useful in the area of tissue repair,

5   where   promotion   of   angiogenesis   is   required. Pharmaceutical compositions containing HA having a molecular weight between about 2×10^6 and about 3×10^6 Daltons can be administered to human or animal subjects to inhibit the formation of hypertrophic scars and

10   keloids arising at wound sites resulting from injury or surgical intervention.

In addition, pharmaceutical compositions containing HA having a molecular weight greater than about 500,000 Daltons can be administered intraarticularly for the

15   treatment of arthritis.

Furthermore, formulations based on HA having a molecular weight greater than about 750,000 Daltons can be used in ophthamology.

The above-described pharmaceutical compositions can

20   be applied topically to the wound site in the form of a liquid, cream, gel, ointment, spray, wound dressing, medicated biomaterial such as a film, gauze, threads, etc., or in the form of an intradermal injection at the time of wound formation or suturing.

25   Doses of high molecular weight HA depend on the individual need of the patient, on the desired effect, and on the route of administration, and can typically be in the range of from about 0.1 mg to about 100 mg per inch or square inch of incision or wound, respectively.

30   The   administration   of   these   pharmaceutical compositions to the wound site can be continued on a daily basis as required until such time as the healing process has been completed in order to avoid scar or keloid formation.

35   The invention being thus described, it is obvious that the same can be modified in various ways. Such modifications are not to be regarded as a departure from

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the spirit and scope of the invention, and all such
modifications that would appear obvious to one skilled
in the art are intended to be included within the scope
of the following claims.

WHAT IS CLAIMED IS:

1.   A process for producing hyaluronic acid *in
vitro*, comprising:
    incubating a protein or protein mixture active in
5   synthesizing hyaluronic acid in a reaction mixture
containing uridine-5'-triphosphate, glucose-1-phosphate,
and N-acetylglucosamine-1-phosphate for a time and under
reaction conditions suitable for the polymerization of
UDP-glucuronic acid and UDP-N-acetylglucosamine to form
10 hyaluronic acid, and suitable for the formation of UDP-
glucuronic acid from UDP and glucose-1-phosphate and the
formation of UDP-N-acetylglucosamine from UDP and
N-acetylglucosamine-1-phosphate; and
    recovering said hyaluronic acid thus produced.

15   2.   The process of claim 1, wherein said protein or
said protein mixture is immobilized on a solid support.

    3.   The process of claim 1, wherein the molecular
weight of said hyaluronic acid thus produced can be
20 varied by varying said time and said reaction
conditions.

    4.   The process of claim 1, wherein the molecular
weight of said hyaluronic acid is in the range of from
about 400 Daltons to about 50,000 Daltons, or from about
25 50,000 Daltons to about 10,000,000 Daltons.

    5.   A process for producing hyaluronic acid *in
vitro*, comprising:
    incubating a protein or protein mixture active in
synthesizing hyaluronic acid in a reaction mixture
30 suitable for the synthesis of hyaluronic acid from UDP-
glucose and UDP-N-acetylglucosamine for a time and under
conditions suitable for the polymerization of UDP-

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glucuronic acid and UDP-N-acetyl-glucosamine to form
hyaluronic acid, and suitable for the formation of UDP-
glucuronic acid from UDP and glucose-1-phosphate and for
the formation of UDP-N-acetylglucosamine from UDP and N-
5   acetylglucosamine-1-phosphate,

        wherein within said reaction mixture, uridine-
5'-triphosphate, glucose-1-phosphate, and N-acetyl-
glucosamine-1-phosphate are contained within a single
hollowfiber, and

10   recovering said hyaluronic acid thus produced.

        6.   A process for producing hyaluronic acid *in
vitro, comprising:

        incubating a protein or protein mixture active in
synthesizing hyaluronic acid in a reaction mixture
15   suitable for the synthesis of hyaluronic acid from
UDP-glucose and UDP-N-acetylglucosamine,

        wherein within said reaction mixture, uridine-
5'-triphosphate and glucose-1-phosphate are contained
within a first hollowfiber, and uridine-5'-triphosphate
20   and N-acetylglucosamine-1-phosphate are contained within
a second hollowfiber,

        wherein   reaction   conditions   in   said   first
hollowfiber are suitable for the formation of UDP-
glucuronic   acid   from   uridine-5'-triphosphate   and
25   glucose-1-phosphate, and wherein reaction conditions in
said second hollowfiber are suitable for the formation
of UDP-N-acetylglucosamine from uridine-5'-triphosphate
and N-acetylglucosamine-1-phosphate, and ·

        recovering hyaluronic acid thus produced.

30   7.   A process for producing hyaluronic acid *in
vitro, comprising:

        incubating   an   immobilized   or   non-immobilized
protein   or   protein   mixture   active   in   synthesizing
hyaluronic acid in alternate cycles with a mixture of
35   uridine- 5'-triphosphate and glucose-1-phosphate, and

separately with a mixture of uridine-5'-triphosphate and
N-acetylglucosamine-1-phosphate, in either order,

wherein each alternate cycle is for a time and
under reaction conditions suitable for the
5   polymerization of UDP-glucuronic acid and UDP-N-acetyl-
glucosamine to form hyaluronic acid,

wherein each alternate cycle is under reaction
conditions suitable for the formation of UDP-glucuronic
acid from uridine 5'-triphosphate and glucose-1-
10   phosphate, and for the formation of UDP-N-acetylglucos-
amine from uridine 5'-triphosphate and N-acetylglucos-
amine-1-phosphate, respectively; and

recovering hyaluronic acid thus produced.

8.   A process for producing hyaluronic acid *in*
15   *vitro*, comprising:

incubating an immobilized or non-immobilized
protein or protein mixture active in synthesizing
hyaluronic acid in alternate cycles with a mixture of
uridine-5'-triphosphate and glucose-1-phosphate, and
20   separately with a mixture of uridine-5'-triphosphate and
N-acetylglucosamine-1-phosphate, in either order,

wherein each alternate cycle is for a time and
under reaction conditions suitable for the
polymerization of UDP-glucuronic acid and UDP-N-acetyl-
25   glucosamine to form hyaluronic acid,

wherein said uridine-5'-triphosphate and glucose-1-
phosphate are contained within a first hollowfiber under
reaction conditions suitable for the formation of
UDP-glucuronic acid,

30   said uridine 5'-triphosphate and N-acetylglucos-
amine-1-phosphate are contained within a second
hollowfiber under reaction conditions suitable for the
formation of UDP-N-acetylglucosamine; and

recovering hyaluronic acid thus produced.

9. The process of any one of claims 7 or 8, wherein the molecular weight of said hyaluronic acid thus produced is varied by varying the number of said alternate cycles.

5   10. The process of any one of claims 7 or 8, wherein said hyaluronic acid thus produced has a molecular weight in the range of from about 400 Daltons to about 50,000 Daltons.

11. The process of any one of claims 1 to 10, wherein said protein or protein mixture active in synthesizing hyaluronic acid is isolated from a naturally occurring or transformed eukaryotic or prokaryotic cell.

12. The process of claim 11, wherein said protein or protein mixture active in synthesizing hyaluronic acid contains hyaluronic acid synthase or a variant form thereof that is active in synthesizing hyaluronic acid.

13. Hyaluronic acid produced by the process of any one of claims 1 to 12, wherein the molecular weight of said hyaluronic acid has a polydispersity of between about 1 and about 2.

14. Hyaluronic acid produced by the process of any one of claims 8 to 10, wherein the molecular weight of said hyaluronic acid has a polydispersity of about 1.

15. Hyaluronic acid produced by the process of any one of claims 1 to 12, wherein said hyaluronic acid is free of any significant quantities of contaminating viruses, proteins, pyrogenic, or inflammatory substances.

16. A pharmaceutical or cosmetic composition

containing hyaluronic acid produced by the process of any one of claims 1-12.

17. Use of hyaluronic acid produced by the process of any one of claims 1-12 for the preparation of a pharmaceutical or cosmetic composition.

18. Use of said pharmaceutical composition of claim 17 in ophthalmology, tissue repair, or rheumatology.

19. The use of claim 18, wherein said pharmaceutical composition is administered topically, intra-articularly, or systemically.

20. A biomaterial containing hyaluronic acid produced by the process of any one of claims 1-12.

21. Use of hyaluronic acid produced by the process of any one of claims 1-12 for the preparation of a biomaterial.

22. The use of claim 10, wherein said biomaterial is selected from the group consisting of a thread, a film, a membrane, a gauze, a sponge, a microsphere, a capsule, a microcapsule, and a device for controlled release of a biologically or pharmaceutically active substance.

23. A process for producing cellulose in vitro, comprising:
    incubating cellulose synthase in a reaction mixture containing uridine-5'-triphosphate and glucose-1-phosphate for a time and under reaction conditions suitable for the synthesis of cellulose, and suitable for the formation of UDP-glucose from uridine-5'triphosphate and glucose-1-phosphate; and

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recovering cellulose thus produced.

24. A process for producing polymannuronic acid *in vitro*, comprising:

   incubating GDP-mannuronic acid polymerase in a
5   reaction mixture containing guanosine-5'-triphosphate
and mannose-1-phosphate for a time and under reaction
conditions suitable for the synthesis of polymannuronic
acid, and suitable for the formation of GDP-mannuronic
acid from guanosine-5'-triphosphate and mannose-1-
10   phosphate, and
   recovering polymannuronic acid thus produced.

25. A process for producing chitin *in vitro*,
comprising:

   incubating chitin synthase in a reaction mixture
15   containing uridine-5'-triphosphate and N-acetylglucos-
amine-1-phosphate for a time and under reaction
conditions suitable for the synthesis of chitin, and
suitable for the formation of UDP-N-acetylglucosamine
from uridine-5'-triphosphate and N-acetylglucosamine-1-
20   phosphate, and
   recovering chitin thus produced.

26. A process for producing a polysaccharide *in vitro*, comprising:

   incubating the sugar-nucleotide synthase or
25   polymerizing enzyme for said polysaccharide in a
reaction mixture containing sugar precursors for said
polysaccharide and nucleotide triphosphate carriers for
said sugar precursors for a time and under reaction
conditions suitable for the synthesis of said
30   polysaccharide, and suitable for the formation of sugar-
nucleotide precursors from said nucleotide triphosphate
carriers and said sugar precursors, and
   recovering said polysaccharide thus produced.

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27.   A polysaccharide produced by the process of
any one of claims 23-26.
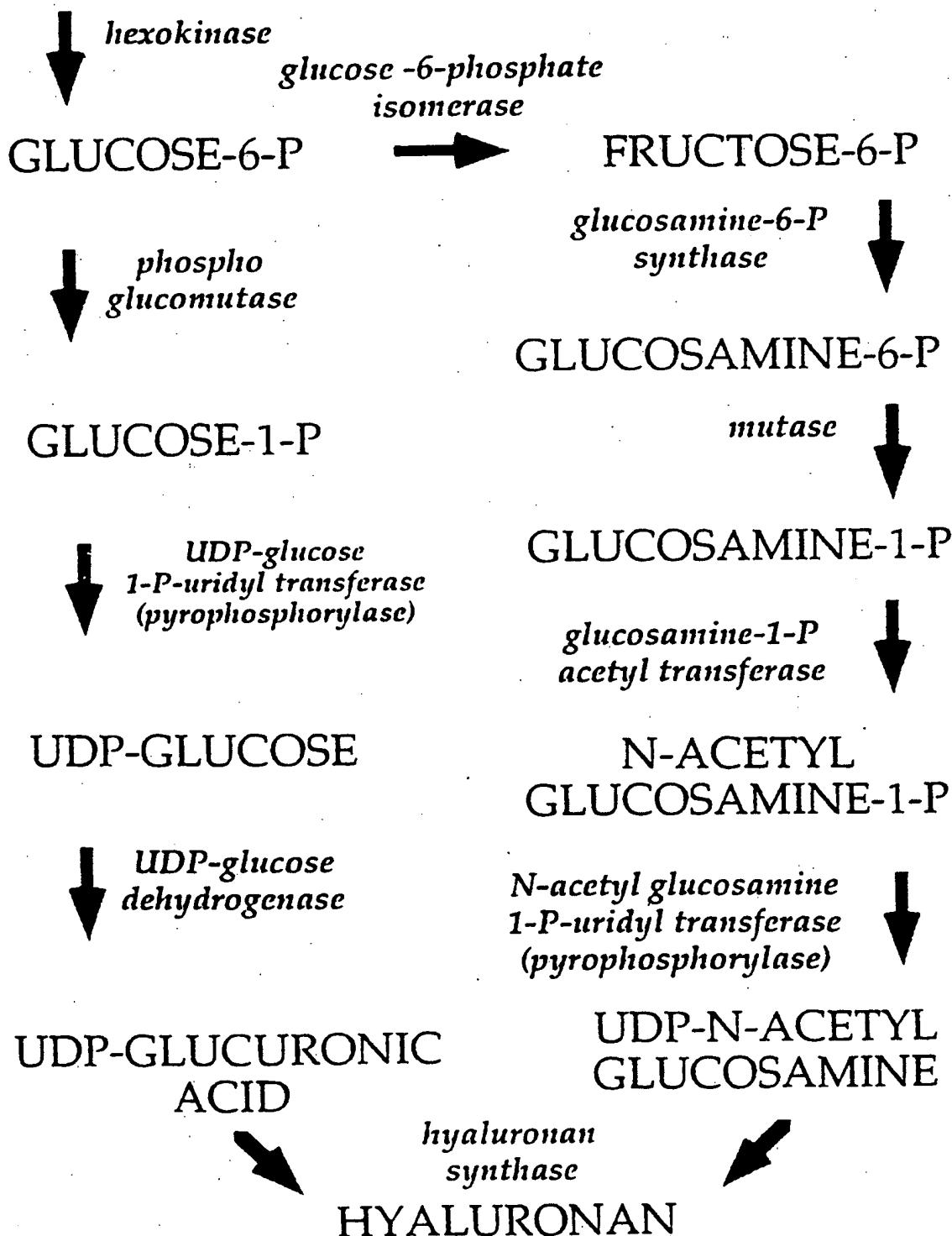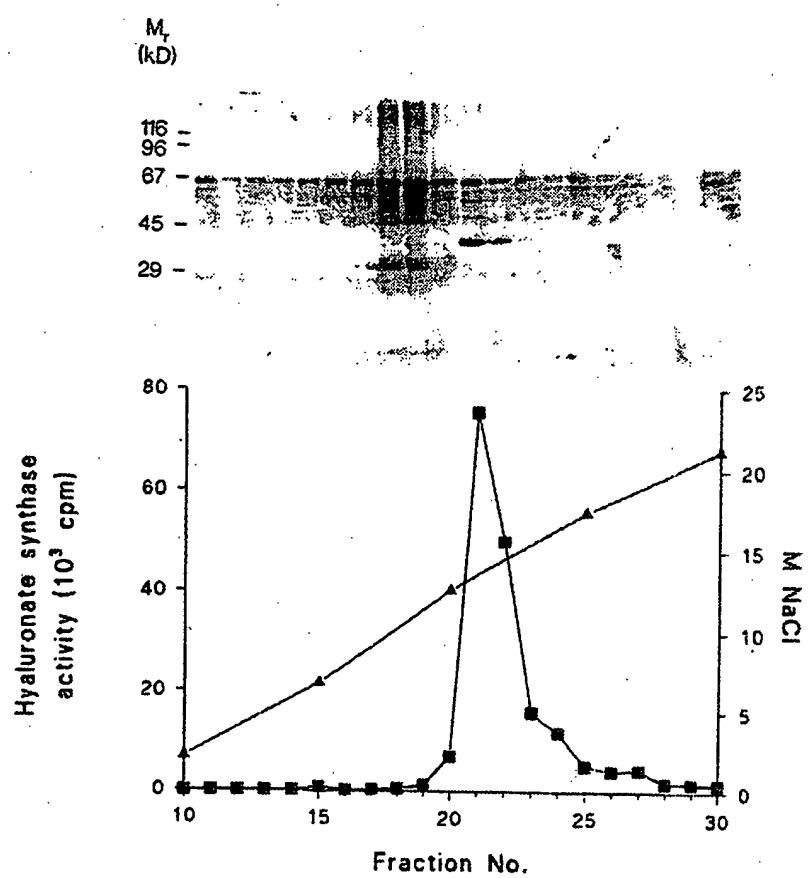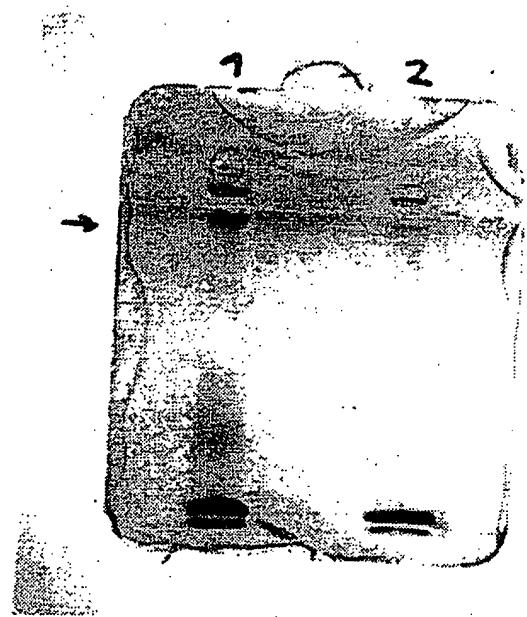
GLUCOSE

↓ *hexokinase*

　　　　　　　　　　glucose -6-phosphate
　　　　　　　　　　　　　isomerase

GLUCOSE-6-P　　→　　FRUCTOSE-6-P

　　　　　　　　　　　　　　glucosamine-6-P　↓
　　　　　　　　　　　　　　　synthase

↓ *phospho*
　glucomutase

　　　　　　　　　　　　　　GLUCOSAMINE-6-P

　　　　　　　　　　　　　　　　mutase ↓

GLUCOSE-1-P

　　　　　　　　　　　　　　GLUCOSAMINE-1-P

↓ *UDP-glucose*
　1-P-uridyl transferase
　(pyrophosphorylase)

　　　　　　　　　　　　　　glucosamine-1-P ↓
　　　　　　　　　　　　　　acetyl transferase

UDP-GLUCOSE

　　　　　　　　　　　　　　N-ACETYL
　　　　　　　　　　　　　　GLUCOSAMINE-1-P

↓ *UDP-glucose*
　dehydrogenase

　　　　　　　　　　　N-acetyl glucosamine
　　　　　　　　　　　1-P-uridyl transferase ↓
　　　　　　　　　　　(pyrophosphorylase)

UDP-GLUCURONIC
　　　ACID

　　　　　　　　　　　　　　UDP-N-ACETYL
　　　　　　　　　　　　　　GLUCOSAMINE

↘　　　*hyaluronan*　　↙
　　　　synthase

HYALURONAN

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

**Enzymatic Synthesis of
Hyaluronic Acid with
Regeneration of Sugar
Nucleotides**
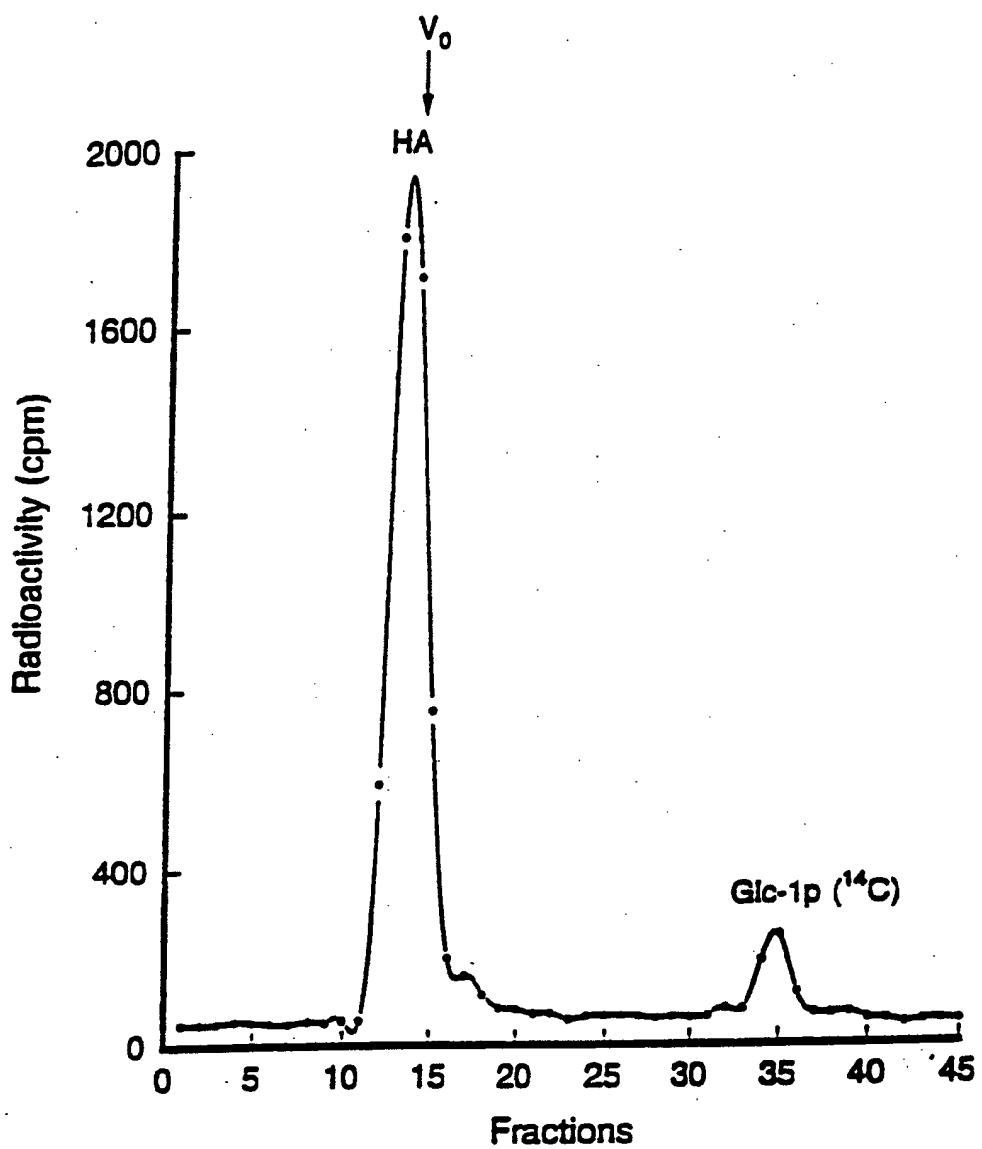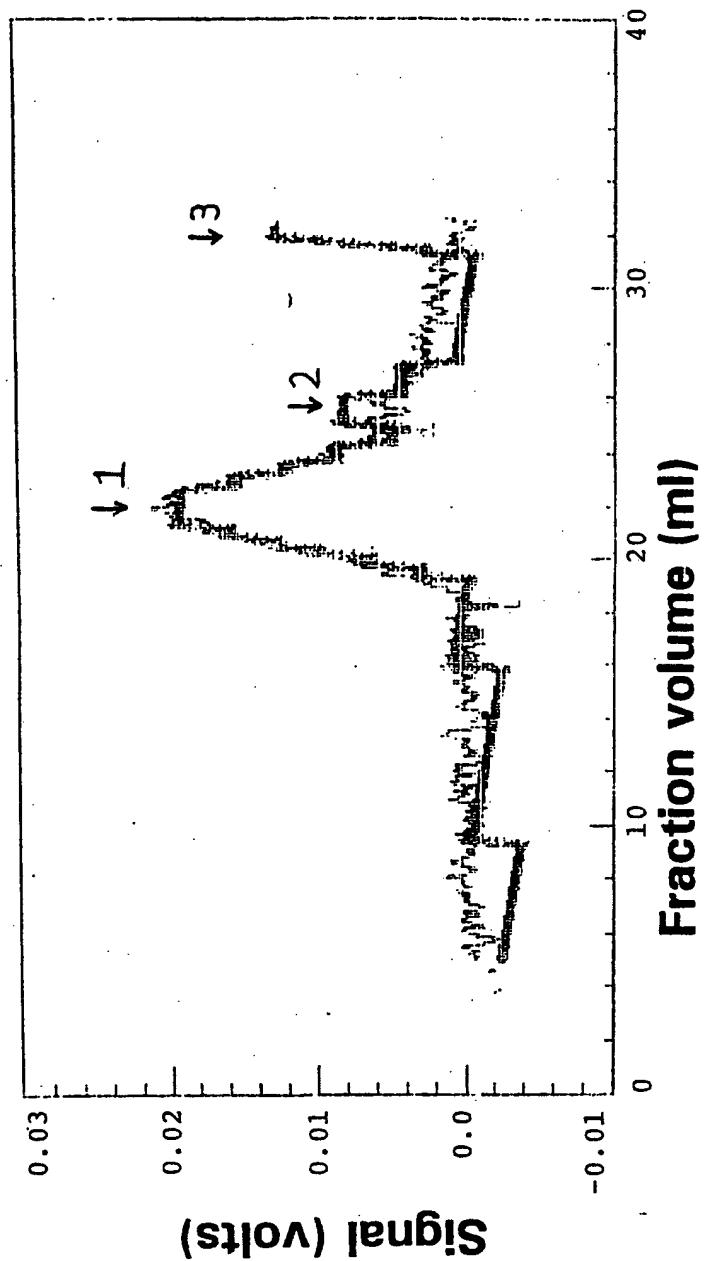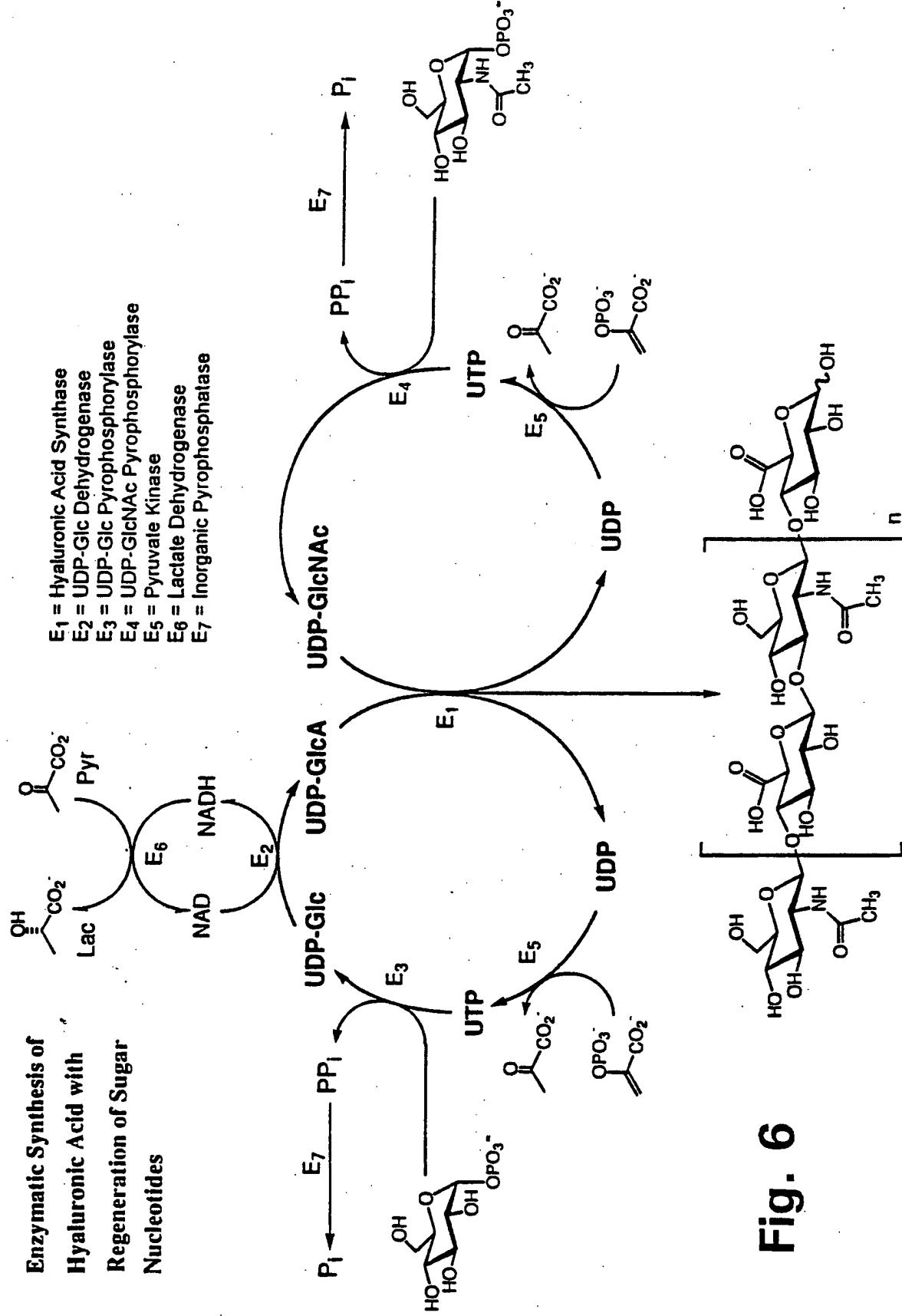
E_1 = Hyaluronic Acid Synthase
E_2 = UDP-Glc Dehydrogenase
E_3 = UDP-Glc Pyrophosphorylase
E_4 = UDP-GlcNAc Pyrophosphorylase
E_5 = Pyruvate Kinase
E_6 = Lactate Dehydrogenase
E_7 = Inorganic Pyrophosphatase

Fig. 6

Hollow Fiber Enzyme Reactor

for the

Synthesis of Hyaluronic Acid

with

Regeneration of Sugar Nucleotides

E_1 = Hyaluronic Acid Synthase
E_2 = UDP-Glc Dehydrogenase
E_3 = UDP-Glc Pyrophosphorylase
E_4 = UDP-GlcNAc Pyrophosphorylase
E_5 = Pyruvate Kinase
E_6 = Lactate Dehydrogenase
E_7 = Inorganic Pyrophosphatase
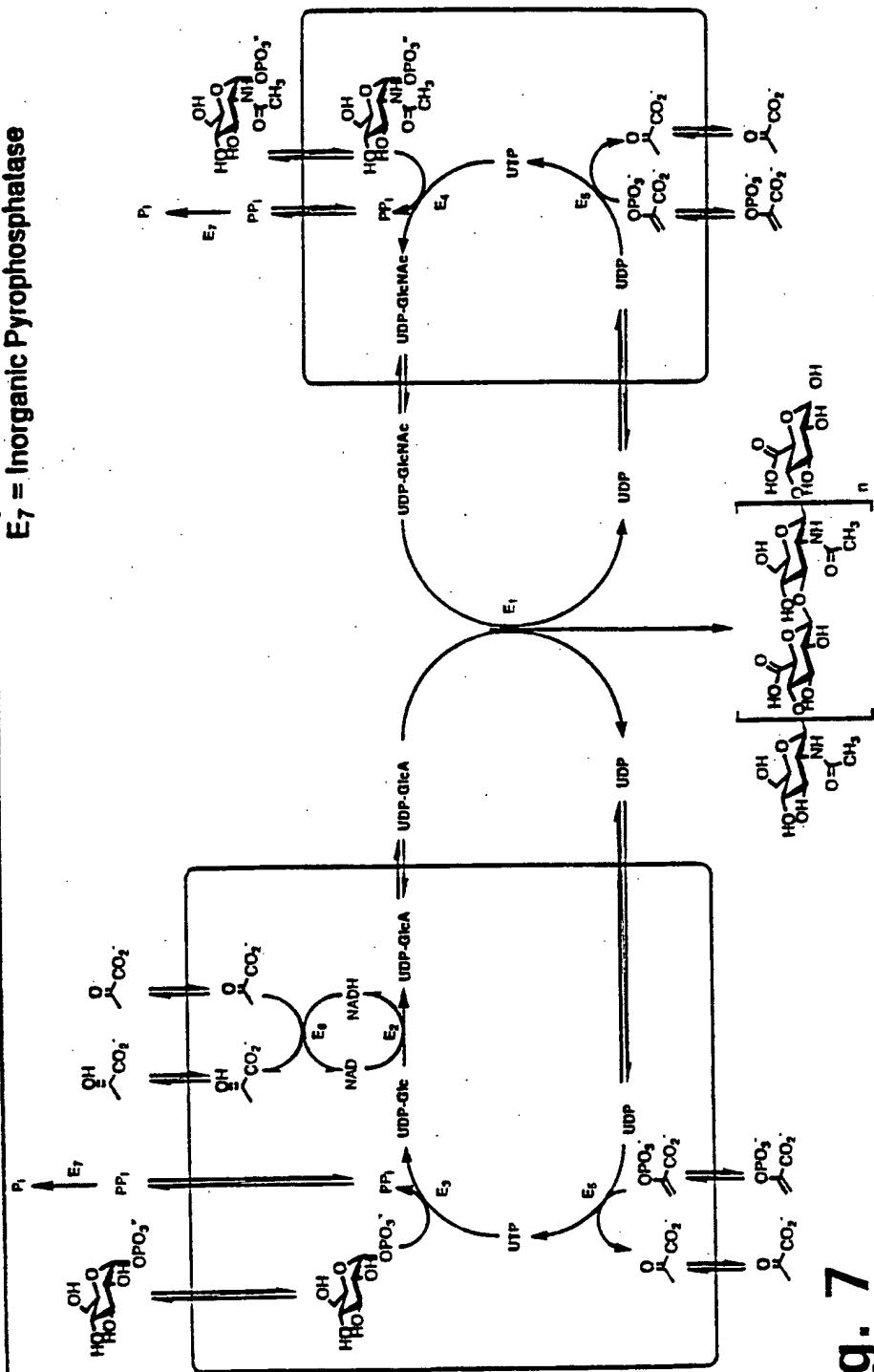
Fig. 7

Enzymatic Synthesis Cellulose with Regeneration of Sugar Nucleotides

E_1 = Cellulose Synth
E_2 = UDP-Glc Pyrophosphorylase
E_3 = Pyruvate Kinase
E_4 = Inorganic Pyrophosphatase

Fig. 8

Enzymatic Synthesis of Polymannuronic
Acid with Regeneration of Sugar
Nucleotides

E₁ = Polymannuronic AcidSynthase
E₂ = GDP-Man Dehydrogenase
E₃ = UDP-Glc Pyrophosphorylase
E₄ = Pyruvate Kinase
E₅ = Lactate Dehydrogenase
E₆ = Inorganic Pyrophosphatase

Fig. 9
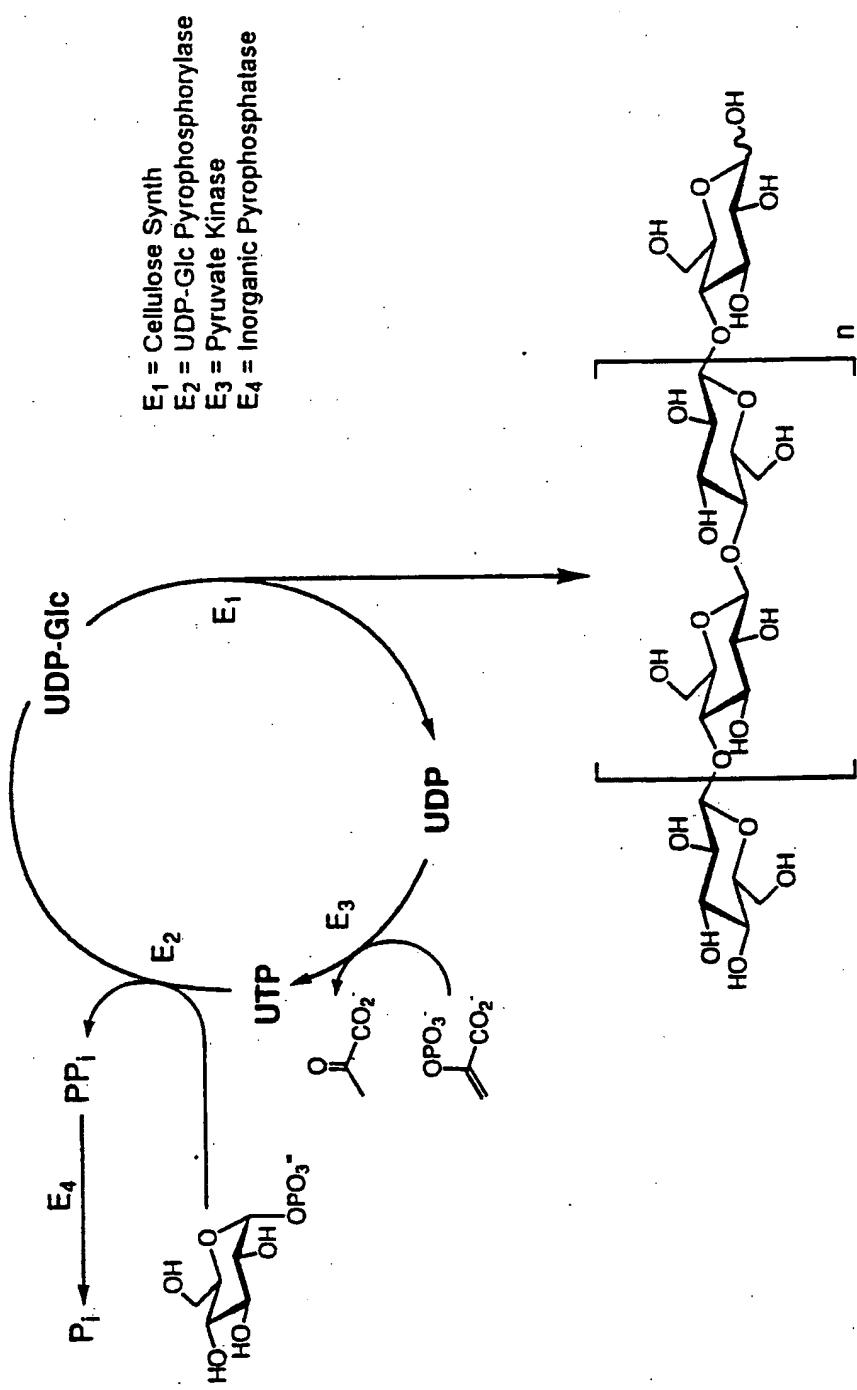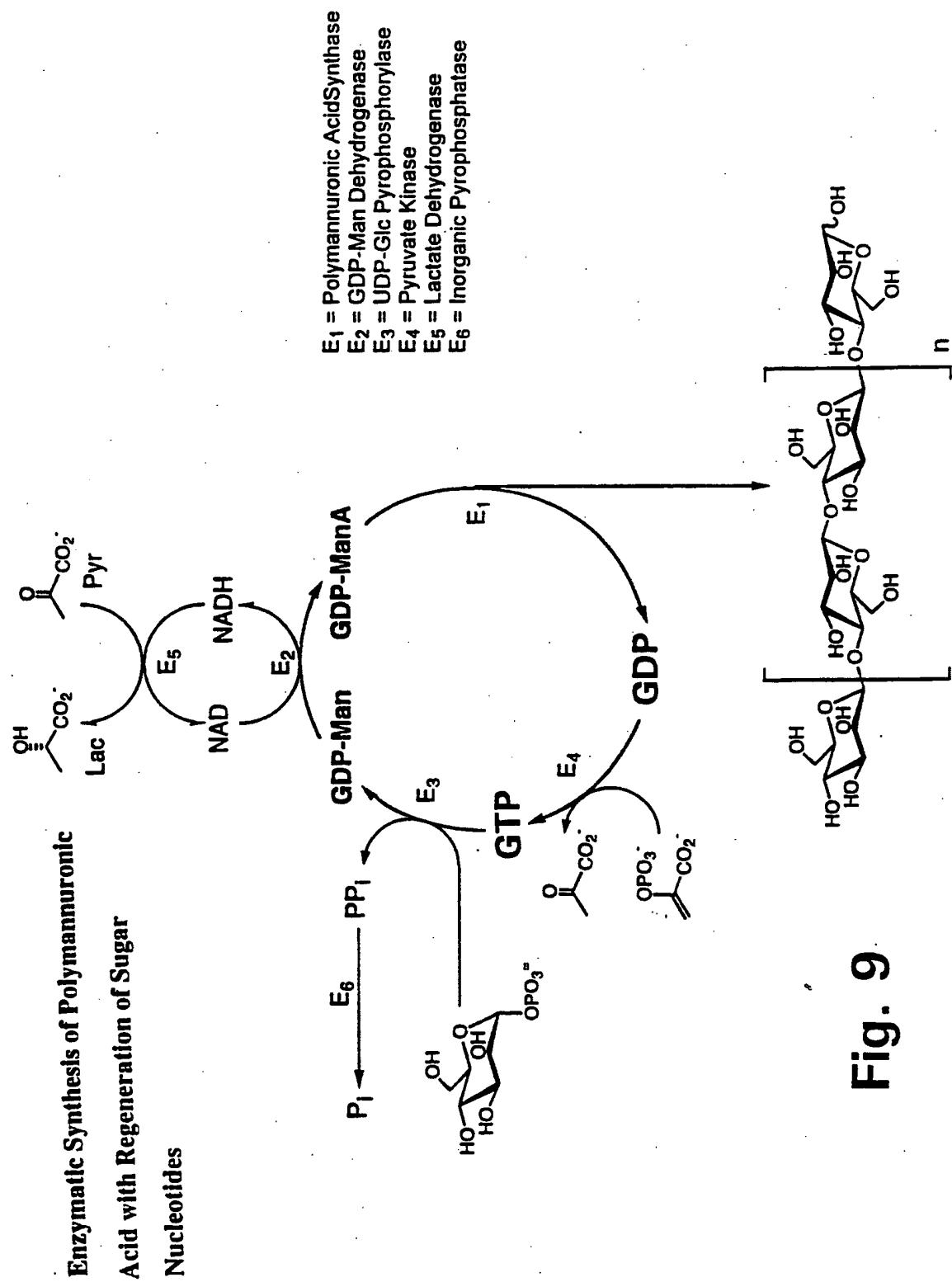
Enzymatic Synthesis of Chitin with Regeneration of Sugar Nucleotides

Fig. 10

E_1 = Chitin Synthase II
E_2 = UDP-GlcNAc Pyrophosphorylase
E_3 = Pyruvate Kinase
E_4 = Inorganic Pyrophosphatase